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A Biochemical Characterization of the Binding of Osteopontin to Integrins $\alpha_v\beta_1$ and $\alpha_v\beta_5$*

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Osteopontin (OPN) is an extracellular matrix protein that binds to integrin $\alpha_v\beta_3$. Here we demonstrate that two other integrins, $\alpha_v\beta_1$ and $\alpha_v\beta_5$, are also receptors for OPN. Human embryonic kidney 293 cells adhere to human recombinant osteopontin (glutathione *S*-transferase-osteopontin; GST-OPN) using integrin $\alpha_v\beta_1$. When the 293 cells are transfected with the β_5 subunit, they can also adhere to GST-OPN using integrin $\alpha_v\beta_5$. Divalent cations regulate the binding of GST-OPN to both $\alpha_v\beta_1$ and $\alpha_v\beta_5$. Mg^{2+} and Mn^{2+} support the binding of GST-OPN to these integrins but Ca^{2+} does not. The highest affinity is observed in Mn^{2+}. In the presence of this ion, the affinity of GST-OPN for $\alpha_v\beta_1$ is 18 nM and the affinity for $\alpha_v\beta_5$ is 48 nM. The antibody 8A2, which is an agonist for β_1, promotes the adhesion of 293 cells to GST-OPN even when Ca^{2+} is present. This observation suggests that cellular events could modulate the affinity of $\alpha_v\beta_1$ for OPN. Collectively, these findings prove that integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ have similar affinity for OPN. Therefore, all three integrins must be considered when evaluating the biological affects of OPN.

Osteopontin (OPN)[1] is a secreted phosphoprotein that was originally isolated from bone (1). OPN is also found in many other fluids and tissues including milk, urine, placenta, kidney, leukocytes, smooth muscle cells, and some tumor cells (for reviews, see Refs. 1 and 2). OPN supports cell adhesion through its Arg-Gly-Asp (RGD) integrin recognition motif. OPN is also rich in aspartic acid residues, and can be heavily glycosylated. The acidic nature of OPN probably accounts for its ability to modulate the growth of calcium crystals in both bone (1, 2) and urine (3).

Integrin $\alpha_v\beta_3$ is the established receptor for OPN. In bone, $\alpha_v\beta_3$ is expressed on osteoclasts and it initiates bone resorption by mediating adhesion of the osteoclast to OPN in bone (4–6). It has also been hypothesized that OPN and integrin $\alpha_v\beta_3$ facilitate vascular remodeling because these two proteins are co-localized in smooth muscle cells following balloon angioplasty (7). Both OPN and integrin $\alpha_v\beta_3$ are also present in human placenta (8, 9), so their interaction could also be relevant to pregnancy.

Although $\alpha_v\beta_3$ is clearly a receptor for OPN, many other integrins also bind the RGD motif (10, 11) and no data have excluded other integrins as receptors for OPN. Therefore, we hypothesized that other integrins with the α_v subunit may also bind OPN. The purpose of this study was to provide a quantitative biochemical analysis of the binding between OPN and integrins $\alpha_v\beta_1$ and $\alpha_v\beta_5$. We reason that a measure of these binding affinities will allow a meaningful comparison with the binding affinity of OPN to $\alpha_v\beta_3$ (12). If more than one integrin does bind OPN with similar affinity, then much information attributing adhesion and signaling events entirely to the interaction between OPN and $\alpha_v\beta_3$ should be re-evaluated.

MATERIALS AND METHODS

Cell Lines—Human embryonic kidney carcinoma 293 cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (Bio Whittaker) supplemented with 10% fetal calf serum (Irvine Scientific), 20 mM Hepes (pH 7.4), 1% glutamine, 1% penicillin, and 1% streptomycin (Sigma). Human integrin subunit β_5 was cloned using polymerase chain reaction and subcloned into the mammalian expression vector pcDNA3 (Invitrogen). Kidney 293 cells were transfected at passage 40 with β_5/pcDNA3 or pcDNA3 vector alone using *N*-[1-(2,3-dioleoyloxy)propl]-*N*,*N*,*N*-trimethylammonium methylsulfate transfection reagent (Boehringer Mannheim). Stable transfectants were obtained after selection in 500 μg/ml G418 (Sigmas) for 2 weeks and maintained thereafter in 250 μg/ml G418. Cells expressing high levels of $\alpha_v\beta_5$ were obtained by sterile FACS with an anti-β_5 monoclonal antibody (mAb), P3G2.

Protein Expression and Purification—In this study a recombinant form of OPN fused the glutathione *S*-transferase (GST-OPN) was used as ligand. We have previously described the characterization of this ligand (12). GST-OPN supports cell adhesion in a manner equivalent to native uropontin, a form of OPN purified from human urine (12). We have also found that both versions of OPN function equally in supporting cell adhesion through integrin $\alpha_v\beta_5$ and $\alpha_v\beta_1$ (data not shown). GST-OPN was chosen in the interest of consistency in performing cell binding studies and because of its availability. Integrin $\alpha_v\beta_5$ was purified from a human placental extract using monoclonal antibody affinity chromatography as described previously (13). The identity and the purity of this protein was assessed by N-terminal amino acid sequencing and by its ability to bind a series of monoclonal antibodies specific for either $\alpha_v\beta_3$ or $\alpha_v\beta_5$.

Vitronectin was purified from human plasma by affinity chromatography on heparin-Sepharose as described (14).

Antibodies—The monoclonal antibody 8A2 and its Fab fragment bind to the integrin β_1 subunit and stimulate the ligand binding function of integrins containing this subunit. An in-depth characterization of this antibody has been published (15, 16). Monoclonal antibody L230 (anti-α_v) was purified from cell culture supernatant from hybridoma cells (ATCC, HB8448) by using protein A-Sepharose. The blocking activity of this antibody has been reported previously (17). Monoclonal antibody P4C10 (anti-β_1) was purchased from Life Technologies, Inc. and was used in ascites form, normally at a dilution of 1:500. Anti-β_1 monoclonal

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[1] The abbreviations used are: OPN, osteopontin; GST-OPN, recombinant osteopontin that is a fusion protein with glutathione *S*-transferase; RGD, Arg-Gly-Asp; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorting.

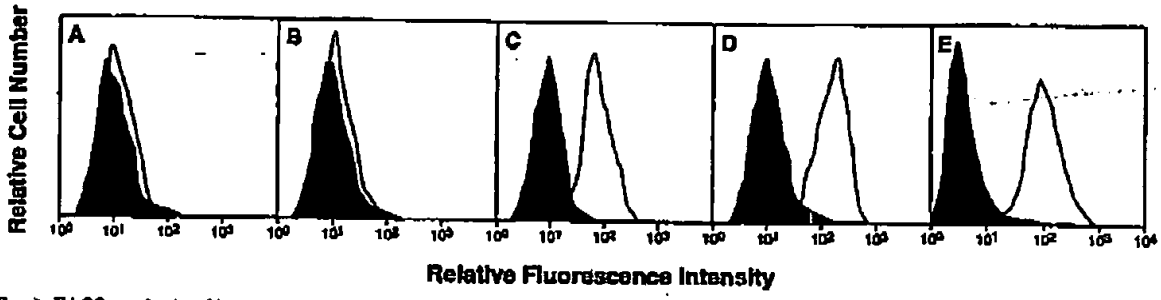

FIG. 1. **FACS analysis of integrin expression on kidney 293 cells.** A panel of monoclonal antibodies was used to assess integrin expression on wild-type and β_5-transfected human kidney 293 cells. Cells were incubated with mouse IgG or with the noted primary antibodies and then with secondary fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Following extensive washing to remove free antibody the cells were analyzed by flow cytometry. The expression level of each integrin subunit is indicated by the mean fluorescence intensity. The integrin expression profile of wild-type 293 cells was analyzed with mAb LM609 against $\alpha_v\beta_3$ (*A*), P3G2 against $\alpha_v\beta_5$ (*B*), 14H4 against α_v (*C*), and mAb 1977 against β_1 (*D*). Following transfection of these cells with the cDNA for β_5 the expression of the $\alpha_v\beta_5$ heterodimer was detected with mAb P3G2 (*E*). Cells transfected with the vector pcDNA3 alone exhibited a profile identical to wild-type 293 cells (not shown).

1977 was purchased from Chemicon Int. Inc.. Monoclonal antibody 6B9 (anti-$\alpha_v\beta_5$) was produced in this laboratory (18). The polyclonal antibody T545 was raised in this laboratory by immunizing rabbits with highly purified integrin $\alpha_v\beta_3$. Prior characterization shows that T545 binds and immunoprecipitates any integrin containing the α_v subunit (data not shown). Nonspecific mouse IgG was obtained from Calbiochem. Monoclonal antibodies LM609 (anti-$\alpha_v\beta_3$) and P3G2 (anti-$\alpha_v\beta_5$) were generously provided by Dr. David Cheresh (The Scripps Research Institute).

Synthetic Peptides—The synthetic peptides with sequence GRGDSP and SPGDRG were purchased from Coast Scientific (La Jolla, CA).

Fluorescence-activated Cell Sorting (FACS)—FACS analysis was performed using standard protocols. Briefly, cells were harvested in phosphate-buffered saline/EDTA, washed once with Dulbecco's modified Eagle's medium, and resuspended in the same media. Cells were incubated with primary antibody for 30 min on ice and then washed twice. Cells were then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Caltag) for 30 min on ice. Cells were washed twice with media and resuspended in phosphate-buffered saline for FACS analysis. FACS analysis was performed on a Becton Dickinson FACSsort.

Cell Adhesion Assays—Cell adhesion was measured as described previously (19). GST-OPN or vitronectin were coated onto 96-well microtiter plates (Titertek) and incubated overnight at 4 °C. Our measurements using ^{125}I-GST-OPN as a tracer indicate that 13–19% of the GST-OPN actually binds the plate when the coating concentration is between 1 and 100 nM. Thus, the amount of ligand available for cell adhesion is considerably less than the coating concentration. There was little variability in coating efficiency so comparisons of cell adhesion as a function of coating concentration are valid. Following exposure to GST-OPN, the plates were then blocked by 30 mg/ml bovine serum albumin in TBS (pH 7.4) for 1 h at 37 °C. Cells were harvested from tissue culture flasks with phosphate-buffered saline/EDTA, washed, and resuspended in adhesion buffer containing 1 × Hanks' balanced salt solution lacking divalent cations, 50 mM Hepes (pH 7.4), 1 mg/ml bovine serum albumin and 0.5 mM Mn^{2+}, 2 mM Ca^{2+}, or 2 mM Mg^{2+}. In most experiments 100 μl of cells (1.5×10^6 cells/ml) were added to each well. Where required, appropriate concentration of agonists (*e.g.* activating mAb 8A2, typically at 1 μg/ml) or antagonists (*e.g.* EDTA at 20 mM or blocking antibodies, 1:500 for ascites and 5–20 μg/ml for purified mAbs) were mixed with the cells before they were added to the wells. Various batches of control ascites gave no inhibition at a 1:500 dilution. After a 45-min incubation at 37 °C, the non-adherent cells were washed off with TBS by gentle aspiration. Adherent cells were detected by a colorimetric assay measuring endogenous cellular lysosomal acid phosphatase activity with a chromophore that absorbs at 405 nm (20). A standard curve with cells in suspension showed that absorbance values were directly proportional to cell number. All experiments were performed at least three times yielding identical results.

Radioligand Binding Measurements—To assess the affinity of GST-OPN for integrins on the 293 cells, binding isotherms of the interaction between ^{125}I-labeled GST-OPN and 293 cells were generated. GST-OPN was radiolabeled with $Na^{125}I$ using IODO-GEN (Pierce Chemical Co.). The specific activity was between 2 and 7×10^{-4} cpm/ng of protein. For binding assays, cells were harvested and resuspended in adhesion buffer containing 0.5 mM Mn^{2+}, which had been found to promote maximal cell adhesion to GST-OPN. A concentration range of ^{125}I-GST-OPN was added to the 293 cells or the β_5-transfected 293 cells (1×10^6 cells/ml) in suspension and the mixture was then incubated for 70 min at 14 °C. At the end of the incubation period, quadruplicate samples of cells (90 μl) were carefully layered onto 20% sucrose cushions (280 μl) in microcentrifuge tubes (West Coast Scientific Inc., Hayward, CA). The tubes were centrifuged for 3 min at 14,000 rpm and the cell pellet in the tip of the tube was amputated and counted in a γ-counter. Nonspecific binding was measured in the presence of 20 mM EDTA and was subtracted from total binding to yield specific binding. All measurements were repeated at least three times yielding identical results.

Bound protein was calculated from the specific activity of the labeled ligand and the results are presented as molecules bound per cell, $[GST\text{-}OPN]_{bound}$. Scatchard plots were derived by plotting $\gamma/[GST\text{-}OPN]_{free}$ against γ, where γ represents $[GST\text{-}OPN]_{bound}$/total number of cells. The binding affinity (K_d) of cell surface integrin for GST-OPN is derived from the slope of this plot. In cases where blocking antibodies were present, preincubation with the antibodies at 14 °C for 15 min was carried out prior to adding ^{125}I-GST-OPN. In cases where binding was stimulated with 8A2, the antibody was added simultaneously with the labeled ligand.

The ability of purified integrin $\alpha_v\beta_5$ to bind GST-OPN was also measured using a solid phase binding assay previously described (19). Purified $\alpha_v\beta_5$ was immobilized on 96-well Titertek microtiter plates at a coating concentration of 50 ng/well. After incubation overnight at 4 °C, nonspecific protein binding sites on the plate were blocked with 30 mg/ml bovine serum albumin and 1 mM of the desired divalent cation(s) in TBS (pH 7.4). Radiolabeled GST-OPN in either 2 mM Ca^{2+} or 0.2 mM Mn^{2+} was then added to the plate. In control wells, nonspecific binding was measured in the presence of a competing RGD peptide. Nonspecific binding was subtracted from the total binding to yield specific binding. Each data point is a result of the average of triplicate wells.

RESULTS

Generating Cell Lines to Study the Binding between OPN and α_v-Integrins—To study the binding of $\alpha_v\beta_1$ and $\alpha_v\beta_5$ to OPN we chose the kidney 293 cells because they lack the $\alpha_v\beta_3$ integrin. These cells do express endogenous $\alpha_v\beta_1$ (21). Thus, the wild-type 293 cells serve as a model for measuring OPN binding to $\alpha_v\beta_1$. To generate a cell line with which we could measure the interaction of $\alpha_v\beta_5$ with OPN, the 293 cells were transfected with the cDNA for β_5. The integrin profile of the wild-type and β_5-transfected 293 cells was compared by flow cytometry (Fig. 1). These studies confirm that the wild-type 293 cells fail to express $\alpha_v\beta_3$ (*panel A*) or $\alpha_v\beta_5$ (*panel B*). The cells express both the α_v and β_1 subunits (*panels C* and *D*). Our immunoprecipitations are consistent with prior studies (21, 22) which indicate that $\alpha_v\beta_1$ is the predominant β_1 containing integrin on these cells (data not shown). Following transfection with the β_5 cDNA the 293 cells also express the $\alpha_v\beta_5$ heterodimer on the cell surface (*panel E*). The cells transfected with the cDNA for β_5 display a 10-fold greater binding of anti-β_5 antibody than the vector transfected or wild-type 293 cells. The expression of

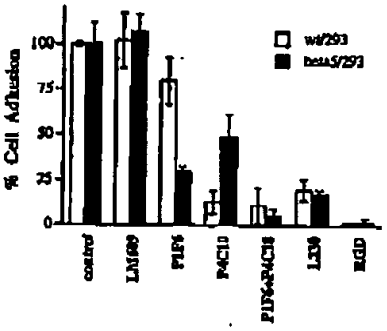

FIG. 2. Wild-type 293 cells and β_5-transfected 293 cells adhere to OPN. The adhesion of wild-type (*open bars*) and β_5-transfected (*dark bars*) 293 cells to GST-OPN was challenged with a series of blocking monoclonal antibodies. Cell adhesion to GST-OPN was performed in the presence of LM609 (anti-$\alpha_v\beta_3$), P1F6 (anti-$\alpha_v\beta_5$), P4C10 (anti-β_1), the mixture of P1F6 and P4C10, L230 (anti-α_v), and RGD peptide as a control inhibitor. The results are expressed as a percentage of control adhesion in the presence of mouse IgG (control). The data are the mean of triplicate wells. *Error bars* denote the standard deviation. This experiment was performed four times yielding identical results.

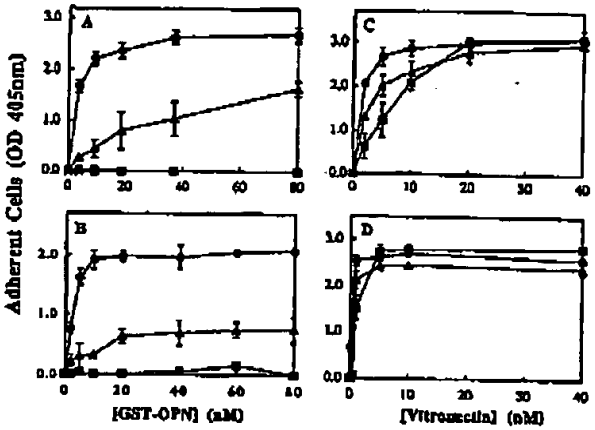

FIG. 3. A comparison of the effects of divalent ions on cell adhesion to osteopontin and vitronectin. The adhesion of kidney 293 cells expressing either integrin $\alpha_v\beta_1$ (*panels A* and *C*) or integrin $\alpha_v\beta_5$ (*panels B* and *D*) to either GST-OPN (*panels A* and *B*) or vitronectin (*panels C* and *D*) was tested in buffer containing Ca^{2+} (■), Mg^{2+} (▲), or Mn^{2+} (●). The adhesion of the β_5-transfected cells was measured in the presence of antibody P4C10 to eliminate any contribution of endogenous $\alpha_v\beta_1$ to cell adhesion. Adhesion assays were conducted as described under "Experimental Procedures." Each data point is the average of quadruplicate measurements. This experiment was performed four times yielding identical results. Additionally, in separate experiments, identical results were obtained when uropontin was used as immobilized ligand.

$\alpha_v\beta_5$ on 293 cells was also confirmed by immunoprecipitation and Western blotting using antibodies specific for $\alpha_v\beta_5$ (data not shown).

Integrins $\alpha_v\beta_1$ and $\alpha_v\beta_5$ are Receptors for OPN—To determine whether $\alpha_v\beta_1$ and $\alpha_v\beta_5$ could mediate cell adhesion to GST-OPN, the wild-type and β_5-transfected 293 cells were allowed to adhere to immobilized GST-OPN. Both cell lines adhere to GST-OPN (Fig. 2). The adhesion was blocked by RGD peptide and by P4C10, an antibody against the β_1 subunit. The adhesion of these cells was also inhibited by L230, an antibody that blocks function of α_v. The antibody against $\alpha_v\beta_3$, LM609, had no effect. Based on these data, and immunoprecipitation experiments showing that the majority of β_1 in these cells is complexed with α_v (data not shown), we conclude $\alpha_v\beta_1$ is a receptor for OPN.

The adhesion of β_5-transfected 293 cells was also blocked by the antibody against the α_v subunit (L230). Approximately 70% of the adhesion of the β_5-transfected cells could be blocked by P1F6, an antibody that interferes with ligand binding to $\alpha_v\beta_5$. The remainder of the adhesion (30%) could be blocked by antibody against the β_1 subunit, indicating that the endogenous $\alpha_v\beta_1$ contributes to the adhesion of these cells to OPN. These experiments show that $\alpha_v\beta_5$ can also mediate cell adhesion to OPN.

The Cation Dependence of Adhesion to OPN Is Distinct from the Cation Dependence for Vitronectin—Ca^{2+} does not support the binding of OPN to integrin $\alpha_v\beta_3$ (12). To determine if Ca^{2+} is similarly ineffective in supporting GST-OPN binding to $\alpha_v\beta_1$ and $\alpha_v\beta_5$, we tested the ability of Ca^{2+}, Mg^{2+}, and Mn^{2+} to support the adhesion of wild-type and β_5-transfected 293 cells to GST-OPN (Fig. 3, *panels A* and *B*). For comparison, the ability of each ion to support the adhesion of each cell line to vitronectin is also shown (*panels C* and *D*). In this study, the amount of coated protein was varied across a concentration range. Each ion was used at a concentration found to support maximal adhesion (not shown). Ca^{2+} did not support adhesion of either cell line to GST-OPN. However, Ca^{2+} did enable maximal cell adhesion to vitronectin. Mn^{2+} was most effective in supporting the adhesion of $\alpha_v\beta_1$ and $\alpha_v\beta_5$ expressing cells to GST-OPN. Mg^{2+}, which is likely to be the physiologically relevant ion, also supported adhesion. Despite slight differences in the rank order potency of divalent ions in supporting adhesion to vitronectin, all three ions did support maximal adhesion to this protein. Physiologic levels of Ca^{2+} supported adhesion to vitronectin but not to GST-OPN. We conclude that there is a fundamental difference in the cation requirement of integrin binding to OPN as opposed to vitronectin.

Measuring the Affinity of GST-OPN for $\alpha_v\beta_1$ and $\alpha_v\beta_5$—The OPN receptor that has received the most attention is integrin $\alpha_v\beta_3$. We recently measured the affinity between GST-OPN and purified integrin $\alpha_v\beta_3$ and found the apparent K_d to be between 5 and 30 nM (12). Recent binding studies between GST-OPN and $\alpha_v\beta_3$ on the surface of M21 melanoma cells has yielded a similar affinity (data not shown). To gauge the significance of the binding of OPN to $\alpha_v\beta_1$ or $\alpha_v\beta_5$, it is important to compare the binding affinities between OPN and each of these integrins. To measure the affinity of OPN for $\alpha_v\beta_1$ and $\alpha_v\beta_5$, we performed binding assays with soluble ^{125}I-GST-OPN. These binding studies were performed by harvesting the wild-type 293 cells or the β_5-transfected 293 cells from tissue culture flasks and placing the cells in suspension. Binding studies were done in Mn^{2+} to obtain the highest affinity between GST-OPN and the two integrins. In the case of the β_5-transfected cells, we found that a small component (typically 10–20% of total binding) of GST-OPN binding was mediated through endogenous $\alpha_v\beta_1$. To eliminate this component from the analysis, the binding studies with the β_5-transfected cells were performed in the presence of a saturating level of a function blocking antibody against the β_1 subunit. Initial control binding studies showed that the specific binding of ^{125}I-GST-OPN to both wild-type and β_5-transfected 293 was inhibited completely by an RGD peptide and by blocking antibody against the α_v subunit (data not shown). To measure the relative affinity of GST-OPN for integrin $\alpha_v\beta_1$ and $\alpha_v\beta_5$, binding isotherms were generated across a concentration range of ^{125}I-GST-OPN (Fig. 4). Scatchard analysis of the binding isotherms revealed that OPN has an affinity of 18 nM for $\alpha_v\beta_1$ (Fig. 4*B*) and 48 nM for $\alpha_v\beta_5$ (Fig. 4*D*). These affinity constants are similar to the apparent K_d (5–30 nM) we measured between GST-OPN and purified $\alpha_v\beta_3$ (12). Consequently, the binding affinity between GST-OPN and all three α_v-integrins is similar.

Binding of GST-OPN to Purified Integrin $\alpha_v\beta_5$—Integrin

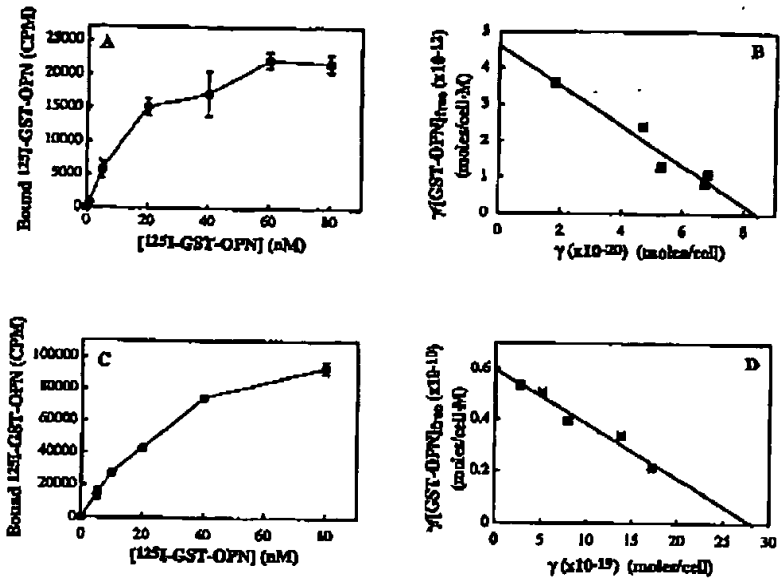

FIG. 4. A measurement of the binding affinity between GST-OPN and $\alpha_v\beta_1$ and $\alpha_v\beta_5$. Isotherms of ^{125}I-GST-OPN binding to wild-type 293 cells (*A*) and β_5-transfected 293 cells (*C*) maintained in suspension were generated. Cells were harvested from tissue culture flasks as usual and were resuspended in adhesion buffer containing 0.5 mM Mn^{2+}. Mn^{2+} was chosen to measure the highest affinity between GST-OPN and the two integrins. ^{125}I-GST-OPN of increasing concentration was added to the cells and the mixture was allowed to incubate with rocking for 70 min at 14 °C. Bound ligand was separated from free ligand by centrifugation through sucrose cushions (see "Experimental Procedures"). Each point is the average of triplicate data points and each isotherm is representative of at least three repetitions. The *error bars* show the standard deviation. To derive the affinity of the interaction between GST-OPN and integrin $\alpha_v\beta_1$ or integrin $\alpha_v\beta_5$ the data shown in *panels A* and *C* were replotted according to the method of Scatchard (53). This derivation yields Scatchard plots for GST-OPN binding to $\alpha_v\beta_1$ (*B*) and $\alpha_v\beta_5$ (*D*). The R^2 values for these lines are 0.87 and 0.90, respectively.

$\alpha_v\beta_5$ is abundant enough in placenta to purify $\alpha_v\beta_5$ for direct binding studies (13). We measured the binding of ^{125}I-GST-OPN to purified $\alpha_v\beta_5$ using the same format that was previously used for $\alpha_v\beta_3$ (12). As shown in Fig. 5*A*, Mn^{2+} is more effective than Ca^{2+} in promoting the binding of OPN to $\alpha_v\beta_5$. Although this assay format does not allow an exact derivation of K_d because the binding of ligand to integrin is irreversible in this assay format (11), we can assign an apparent K_d and compare this value to that obtained for $\alpha_v\beta_3$. In Mn^{2+}, the apparent K_d of GST-OPN for $\alpha_v\beta_5$ is 20 nM, which is comparable to the value of 5–30 nM for $\alpha_v\beta_3$ (12). Thus, the two purified integrins bind GST-OPN with nearly equal affinity. The purified $\alpha_v\beta_5$ is obtained from a placental lysate by first depleting the lysate of $\alpha_v\beta_3$ by affinity chromatography. Therefore, we performed an enzyme-linked immunosorbent assay on the purified $\alpha_v\beta_5$ to make sure that it contained no contaminating $\alpha_v\beta_3$. This enzyme-linked immunosorbent assay was done with mAb 6B9 which is specific for $\alpha_v\beta_5$ (18) and mAb LM609 which binds only to $\alpha_v\beta_3$. As shown in Fig. 5*B*, the purified $\alpha_v\beta_5$ contains no detectable $\alpha_v\beta_3$, proving that OPN binds to purified $\alpha_v\beta_5$.

Adhesion to OPN through Integrin $\alpha_v\beta_1$ Can Be Stimulated by Activation of the β_1 Subunit with Monoclonal Antibody 8A2—It has been reported that many integrins can exist in multiple affinity states (16, 24–30). These observations indicate that there may be cellular pathways that control the affinity of an integrin for its ligand. Because our data shows that Ca^{2+} does not support adhesion to OPN, we wondered if other stimuli could override this phenomena. Since the physiologic stimuli that regulate integrin affinity have not been completely discerned, we made use of the monoclonal antibody 8A2. This antibody is a known agonist for β_1 integrins (15, 16) and it has been suggested that 8A2 mimics the physiologic activation of these integrins. We tested the ability of 8A2 to stimulate the adhesion of 293 cells to OPN. These studies were performed in buffer containing Ca^{2+}. As shown in Fig. 6*A*, 293 cells adhered to GST-OPN in the presence of mAb 8A2 in buffer containing Ca^{2+}. No adhesion to GST-OPN was observed in the presence of normal mouse IgG in the same buffer. To determine whether this stimulation was saturable and corresponded with the binding of 8A2 to $\alpha_v\beta_1$, the number of cell surface binding

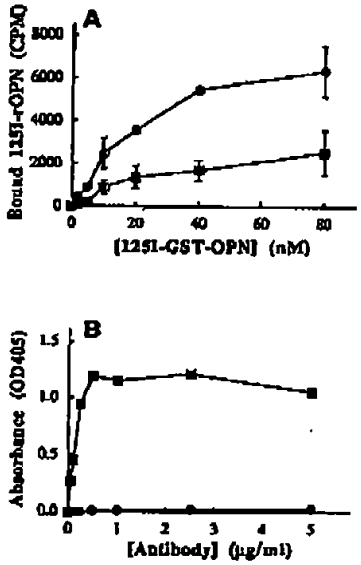

FIG. 5. Integrin $\alpha_v\beta_5$ is also a receptor of osteopontin. *A*, the binding of GST-OPN to integrin $\alpha_v\beta_5$ was also determined by a solid phase binding assay. This study was done in buffer containing Mn^{2+} (0.2 mM, ●) or Ca^{2+} (2 mM, ■) as divalent cation. The binding assay was performed as described previously (19). The data are the average of triplicate points in which the error was less than 12% of the total binding. Nonspecific binding was less than 8% of the total binding as determined by incubation with competing RGD peptide. Nonspecific binding is subtracted from the total binding. *B*, to ensure that no contaminating $\alpha_v\beta_3$ was present in the $\alpha_v\beta_5$ preparation, an enzyme-linked immunosorbent assay was performed. The monoclonal antibody 6B9 (■) (18) was used as a probe of integrin $\alpha_v\beta_5$ and antibody LM609 (●) was used to detect integrin $\alpha_v\beta_3$.

sites for the antibody was measured. As shown in Fig. 6*B* the binding of ^{125}I-mAb 8A2 to 293 cells in suspension approaches saturation between 0.5 and 1 μg/ml of antibody. This concentration corresponds closely with the amount of the antibody that maximally stimulates adhesion to OPN (Fig. 6*A*). From the Scatchard plot shown in Fig. 6*C*, the K_d of mAb 8A2 for $\alpha_v\beta_1$

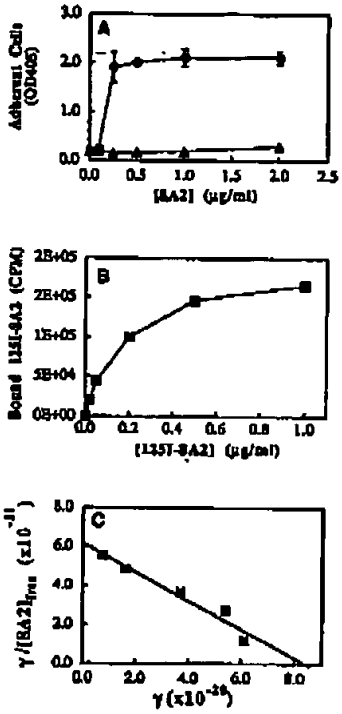

FIG. 6. Antibody 8A2 stimulates 293 cell adhesion to OPN in Ca^{2+}. *A*, the adhesion of wild-type 293 cells to GST-OPN was measured in the presence of a range of mAb 8A2 (●) or normal mouse IgG ( ). Cells were resuspended in adhesion buffer containing 2 mM Ca^{2+}. The cells (100 μl at 1.5×10^6 cells/ml) were allowed to adhere to GST-OPN at a coating concentration of 10 nM. The data are the mean of triplicate wells. *Error bars* denote the standard deviation. This experiment was performed three times yielding identical results. *B*, the affinity and number of binding sites on 293 cells for mAb 8A2 was measured by generating a binding isotherm with radiolabeled 8A2. Nonspecific binding was determined by competition with an excess of unlabeled 8A2 and was typically less than 10% of total binding. The specifically bound counts are shown. *C*, these data were transformed into a Scatchard plot (53) to quantify the binding affinity and the number of binding sites.

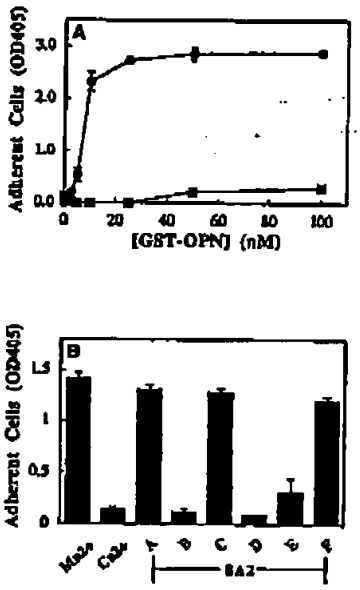

FIG. 7. mAb 8A2 simulates adhesion to GST-OPN through integrin $\alpha_v\beta_1$. *A*, the adhesion of wild-type 293 cells to GST-OPN was measured in the presence of a range of coated GST-OPN in the presence of 1 μg/ml of either 8A2 (●) or normal mouse IgG (■). Cells (100 μl at 1.5×10^6 cells/ml) were resuspended in adhesion buffer containing 2 mM Ca^{2+} and were allowed to adhere to a range of GST-OPN for 45 min at 37 °C. The data are the mean of triplicate wells. *Error bars* denote the standard deviation. *B*, to confirm that integrin $\alpha_v\beta_1$ is mediating 8A2-stimulated adhesion to GST-OPN in Ca^{2+}, the adhesion was challenged by synthetic peptides and monoclonal antibodies. These are: mAb 8A2 only (*A*), 100 μM GRGDSP (*B*), 100 μM SPDGRG (*C*), 1:500 dilution of anti-β_1 ascites P4C10 (*D*), 20 μg/ml of anti-α_v mAb L230 (*E*), and 20 μg/ml nonspecific mouse IgG (*F*).

on 293 cells is 1.4 nM and the number of cell surface binding sites is 51,000. This value matches exactly the number of $\alpha_v\beta_1$ molecules on the cell surface as measured by binding of ^{125}I-GST-OPN (Fig. 4, *A* and *B*).

We also examined the ability of mAb 8A2 to stimulate cell adhesion across the range of coated GST-OPN (Fig. 7*A*). In the presence of mAb 8A2, the coating concentration of GST-OPN that support half-maximal cell adhesion is similar to that obtained in Mn^{2+} (Fig. 3*A*), indicating that both 8A2 and Mn^{2+} induce the high affinity state of $\alpha_v\beta_1$. To verify that mAb 8A2 stimulates adhesion to OPN by enhancing the affinity state of $\alpha_v\beta_1$, adhesion assays were done in the presence of mAb 8A2 and a series of antagonists, including RGD peptide, antibody P4C10 (anti-β_1), and mAb L230 (anti-α_v). The adhesion to GST-OPN that is induced by mAb 8A2 can be blocked by each of the above inhibitors (Fig. 7*B*). Neither random peptide nor mouse IgG affected cell adhesion. Several other control experiments were also performed. These studies showed that the Fab fragment of mAb 8A2 was as effective as the whole antibody and that mAb 8A2 did not induce the expression of more $\alpha_v\beta_1$ on the cell surface.

DISCUSSION

Many interactions between cells and the extracellular matrix depend on cellular recognition of the RGD motif within adhesive proteins. Small peptides with the RGD sequence will bind to several integrin adhesion receptors, but larger adhesive proteins display considerable integrin binding specificity. Therefore, an important issue with every RGD-containing adhesive protein is to identify its receptor(s). OPN, for instance, binds to integrin $\alpha_v\beta_3$, but not to the platelet integrin $\alpha_{IIb}\beta_3$ (12). However, it is now apparent that several integrins have ligand binding properties similar to $\alpha_v\beta_3$, these are the four other integrins containing the α_v subunit, $\alpha_v\beta_1$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$ (22). Like $\alpha_v\beta_3$, two of these integrins, $\alpha_v\beta_1$ and $\alpha_v\beta_5$, bind to vitronectin. This functional similarity lead us to suspect that both of these integrins may also bind OPN. Since both $\alpha_v\beta_1$ and $\alpha_v\beta_5$ have been identified in tissues, like bone and the vasculature where OPN is involved in tissue remodeling (1, 2, 31), there is the potential for a physiologically relevant interaction between these integrins and OPN.

Ideally experiments designed to characterize the interactions between integrins and their ligands would provide a quantitative measure of these interactions so that a hierarchy of binding affinities is available. Here, the affinity between OPN and integrin $\alpha_v\beta_1$ and $\alpha_v\beta_5$ was determined by measuring the binding of ^{125}I-GST-OPN to these integrins present on the surface of kidney 293 cells. Scatchard analysis shows that in the highest affinity state, the K_d of GST-OPN is 18 nM for $\alpha_v\beta_1$ and 48 nM for $\alpha_v\beta_5$. We also measured the apparent affinity between GST-OPN and purified integrin $\alpha_v\beta_5$. It was impossible to determine a K_d using Scatchard analysis because GST-OPN binding to $\alpha_v\beta_5$ immobilized in microtiter wells was non-dissociable. This non-dissociable binding has been observed previously with integrin $\alpha_v\beta_3$ and its potential physiologic significance has been discussed (23). Despite this binding anom-

aly, the apparent K_d (20 nM) between GST-OPN and purified integrin $\alpha_v\beta_5$ is comparable to the affinity between GST-OPN and purified $\alpha_v\beta_3$ measured in the same assay under the same conditions (12). In addition, several cell adhesion experiments showed that the coating concentration of GST-OPN necessary for half-maximal cell adhesion through $\alpha_v\beta_1$, $\alpha_v\beta_5$ (Fig. 3, *A* and *B*), and $\alpha_v\beta_3$ (12) was identical. Collectively, our data suggest there is no substantial preference in the binding of OPN to any of these α_v-integrins. It is important to reiterate that OPN does not bind to all integrins. We recently measured the binding of OPN to the platelet integrin $\alpha_{IIb}\beta_3$ and showed that these two proteins do not interact (12).

The binding of OPN to its different α_v-integrin receptors is also similar with respect to divalent ion requirement. We previously found that both Mg^{2+} and Mn^{2+} support OPN binding to integrin $\alpha_v\beta_3$, but that Ca^{2+} suppresses this interaction (12). Here, we show that Ca^{2+} also fails to support the binding of OPN to integrins $\alpha_v\beta_1$ and $\alpha_v\beta_5$. This observation is important because it illustrates a key difference between the binding of OPN and vitronectin to α_v-integrins. Although small differences exist in the rank-order potency of divalent ions in supporting adhesion to vitronectin, physiologic levels of Ca^{2+} supported maximal cell adhesion to this protein through $\alpha_v\beta_1$ and $\alpha_v\beta_5$. This is in contrast to the adhesion to OPN which is not supported at any level by Ca^{2+}. In this regard it is worth noting an important biochemical distinction between vitronectin and OPN. The vitronectin used in these studies is a multimer, often containing between 12 and 15 vitronectin moieties per multimer (32, 33). There is substantial evidence that the multimeric vitronectin is also present in extracellular matrices *in vivo* (32–34). In contrast, the OPN used in these studies was proven to be monomeric by mass spectral analysis (12) and gel filtration chromatography (data not shown). The soluble OPN found in body fluids is also assumed to be a monomer. Consequently, it is possible that multimeric vitronectin engages several integrins simultaneously, thereby overriding an otherwise lower affinity between vitronectin and α_v-integrins in calcium ion.

While Ca^{2+} does not support OPN binding to integrins $\alpha_v\beta_1$ and $\alpha_v\beta_5$, Mn^{2+} is able to enhance the binding. This result is not unexpected because Mn^{2+} is known to activate ligand binding functions of many integrins (22, 35–38). The physiologic activation of integrins can also be mimicked by monoclonal antibodies (16, 39–41). For example, several studies have demonstrated that integrins can be subject to physiologic activation. The best example is the platelet fibrinogen receptor integrin $\alpha_{IIb}\beta_3$, which exists in a dormant state on resting platelets. This integrin responds to platelet activation by increasing its affinity for soluble fibrinogen (42). This increased binding affinity enables platelet aggregation at the site of a wound. Our data indicate that the binding of GST-OPN to integrin $\alpha_v\beta_1$ can be enhanced by both Mn^{2+} and the mAb 8A2, which is known to be an agonist of other β_1-integrins. Although several other integrins are known to have agonists other than divalent ions (16), to our knowledge, this is the first demonstration that the affinity of an α_v-integrin can be modulated by an agonist besides Mn^{2+}. By analogy with other integrins that are similarly stimulated, it is possible that this artificial stimulus indicates the potential for enhancing the affinity state of the integrin by physiologic means. It is important to emphasize that even when Ca^{2+} is present, the mAb 8A2 was able to enhance cell adhesion to OPN to maximal levels. Thus, the suppressive effects of Ca^{2+} can be overridden by other stimuli. In future studies, it will be important to determine if $\alpha_v\beta_3$ and $\alpha_v\beta_5$ can be similarly stimulated to bind OPN when Ca^{2+} is present and to determine if there are cellular signals that can promote adhesion to OPN in Ca^{2+}.

The binding of OPN to $\alpha_v\beta_1$ and $\alpha_v\beta_5$ may be important to bone homeostasis. OPN is thought to be one of the most important matrix proteins for osteoclast adhesion (2, 4). In addition, soluble OPN stimulates intracellular signaling in osteoclasts, including Ca^{2+} fluxes and the phosphorylation of intracellular proteins (43). It has been reported that integrin $\alpha_v\beta_1$ is present on human osteoclasts (44–47) and that integrin $\alpha_v\beta_5$ is present on chicken osteoclast precursors (48, 49). Therefore both of these integrins are positioned to mediate interactions between OPN and cells in bone. Our finding that integrins $\alpha_v\beta_1$ and $\alpha_v\beta_5$ have high affinity for OPN indicates that interactions between OPN and these receptors may play an essential role in bone remodeling. Blocking the activity of $\alpha_v\beta_3$ with antibodies inhibits bone resorption, but no analogous study has been done with antagonists of other α_v-integrins. Our data suggest that similar experiments should be done with antagonists of β_1 and β_5.

Recent study also indicates that OPN is involved in vascular injury and repair (6, 31). One of the initial responses to vascular injury is the formation of a neointima which precedes the formation of atherosclerotic lesions (50). Giachelli *et al.* (51) recently showed that OPN expression is increased substantially in both rat and human smooth muscle cells surrounding a vessel that has been exposed to a catheter-induced injury. Because of the temporal regulation of OPN synthesis following this insult, the hypothesis was put forth that the OPN expressed by smooth muscle cells may be an important modulator of cell migration and proliferation associated with neointima formation (7, 52). The same group showed that integrin $\alpha_v\beta_3$ mediates only a portion of smooth muscle cell or to OPN; a major component of this adhesion was not blocked by antagonists specific for $\alpha_v\beta_5$ (7). The data presented in this report indicate that integrins $\alpha_v\beta_5$ and $\alpha_v\beta_1$ should be considered as candidate OPN receptors involved in guiding vascular repair.

The kinetic data in this report provide information essential to an understanding of the biology of OPN. Many adhesive and signaling events are tied to cellular exposure to OPN. In large part, it had been assumed that these events are mediated by integrin $\alpha_v\beta_3$ because it was the only known OPN receptor. In conjunction with our prior study (12), the data in this report show that $\alpha_v\beta_3$, $\alpha_v\beta_1$, and $\alpha_v\beta_5$ have similar affinities for OPN and that the ion regulation of OPN binding to each integrin is nearly identical. Therefore, along with $\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$ must now be considered receptors for OPN.

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